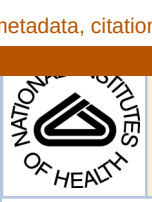

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L-Selectin Is Dispensable for T Regulatory Cell Function Postallogeneic Bone Marrow Transplantation

M. J. Carlson[a], **L. M. Fulton**[a], **J. M. Coghill**[a], **M. L. West**[a], **J. E. Burgents**[a], **Y. Wan**[a], **A. Panoskaltsis-Mortari**[b], **T. F. Tedder**[c], **B. R. Blazar**[b], and **J. S. Serody**[a,*]

[a]Departments of Medicine, Microbiology, and Immunology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC

[b]Department of Pediatrics, Division of Blood and Marrow Transplantation and University of Minnesota Cancer Center, Minneapolis, MN

[c]Department of Immunology, Duke University Medical Center, Durham, NC

Abstract

In murine models, the adoptive transfer of $CD4^+/CD25^+$ regulatory T cells (T_{regs}) inhibited graft-versus-host disease (GvHD). Previous work has indicated a critical role for the adhesion molecule L-selectin (CD62L) in the function of T_{regs} in preventing GvHD. Here we examined the capacity of naive wild-type (WT), $CD62L^{-/-}$ and *ex vivo* expanded $CD62L^{Lo}$ T_{regs} to inhibit acute GvHD. Surprisingly, we found that $CD62L^{-/-}$ T_{regs} were potent suppressors of GvHD, whereas $CD62L^{Lo}$ T_{regs} were unable to inhibit disease despite being functionally competent to suppress allo T cell responses *in vitro*. Concomitant with improved outcomes, WT and $CD62L^{-/-}$ T_{regs} significantly reduced liver pathology and systemic pro-inflammatory cytokine production, although $CD62L^{-/-}$ T_{regs} were less effective in reducing lung pathology. While accumulation of $CD62L^{-/-}$ T_{regs} in GvHD target organs was equivalent to WT T_{regs}, $CD62L^{-/-}$ T_{regs} did not migrate as well as WT T_{regs} to peripheral lymph nodes (PLNs) over the first 2 weeks posttransplantation. This work demonstrated that CD62L was dispensable for T_{reg}-mediated protection from GvHD.

Keywords

Allogeneic stem cell transplantation; CD62L; GvHD; regulatory T cells

Introduction

Allogeneic hematopoeitic stem cell transplantation (allo-HSCT) is a potentially curative therapy for numerous blood born-malignant and nonmalignant disorders (1,2). Although allo-HSCT holds much promise, the prevalence of graft-versus-host disease (GvHD) limits its widespread use (3). $CD4^+/CD25^+$ naturally occurring T regulatory (T_{reg}) cells offer a potential solution to the prevention of GvHD. Importantly, T_{regs} can suppress allo-reactive T

*Corresponding author: Jonathan S. Serody, Jonathan_Serody@med.unc.edu.

Disclosure

The authors of this manuscript have no conflict of interest to disclose as described by the *American Journal of Transplantation*.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: $CD62L^{Lo}$ T_{regs} are potent suppressers *in vitro*

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cell responses, including those involved in solid organ and skin allograft rejection (4). Multiple groups, including our own, have demonstrated that T_{regs} are capable of inhibiting GvHD without impacting the GvL response (5,6).

L-selectin (CD62L) is a member of the selectin family that is involved in leukocyte homing (7). CD62L is constitutively expressed by myeloid cells, nave lymphocytes and central memory T cells (7,8). We, and others, have shown the importance of T_{reg} homing molecule expression in preventing GvHD (5,9,10). These studies provided evidence that the phenotype of the $CD62L^{Hi}$ T_{reg} population was responsible for inhibition of GvHD; however, they did not directly assess the role of CD62L in this process. Decreased expression of CD62L may be indicative of a T_{reg} subset that is biologically distinct from $CD62L^{hi}$ T_{regs} independent of the function of CD62L.

Using a clinically relevant model of allo-HSCT we now show that CD62L expression by T_{regs} was not required for the inhibition of GvHD as $CD62L^{-/-}$ T_{regs} provided similar protection from lethal acute GvHD compared to WT T_{regs}. In addition, CD62L expression was not critical for T_{reg} migration to GvHD target organs. However, the expression of CD62L was important for the prompt migration of T_{regs} to PLNs.

Methods

Mice

Donor mice consisted of male C57BL/6J (B6), ($H\text{-}2^{b}$; The Jackson Laboratory, Bar Harbor, ME), $Thy1.1^{+}$ mice ($H\text{-}2^{b}$; The Jackson Laboratory) and $CD62L^{-/-}$ mice, which have been described previously (11). $CD62L^{-/-}$ mice were crossed with B6 mice expressing the enhanced GFP (eGFP) protein to generate eGFP-expressing $CD62L^{-/-}$ mice. The generation of B6-eGFP mice has been described (12). In some experiments T_{reg} cells were isolated from FIR mice (expressing red fluorescent protein (RFP) under the FoxP3 promoter) as described (13). Recipient mice were male (C57BL/6JXDBA/2) FI mice, (B6D2) ($H\text{-}2^{bxd}$; The Jackson Laboratory).Within each experiment, all recipient and donor mice were male mice ranging from 9 to 14 weeks. All animal experiments were performed in accordance with protocols approved by the University of North Carolina Institutional Animal Care and Use Committee.

Antibodies and flow cytometry

Antibodies with the following specificities were purchased from eBiosciences (San Diego, CA): anti-CD4 (RM4.5), CD62L (Mel-14), CD25 (PC61), CD8 (53-6-7), Thy1.1 (HIS51) and FoxP3 (FJK-16s). Acquisition was performed on a FACSCalibur using CellQuest software (BD Biosciences; San Jose, CA). Analysis was performed using FlowJo (Treestar Inc., Ashland, OR) software.

Preparation of cells for transplant and bone marrow transplants

T cell-depleted bone marrow (TCD BM) cells, T_{eff} cells and T_{regs} were isolated and infused as described (14).

T_{reg} cell expansion

$CD4^{+}/CD25^{+}/RFP^{+}/CD62L^{high}$ cells were sorted on a MoFlo cell sorter (Dako A/S, Glostrup, Denmark) from the spleens of RFP-FoxP3 mice. Sorted cells were expanded with plate-bound anti-CD3 (145-2C11, 15μg/mL; eBioscience) and CD28 (37.51, 10μg/mL; eBioscience) supplemented with IL-2 (500 units/mL; Peprotech; Rocky Hill, NJ) for 12 days. After 12 days, cells were recovered, stained for CD62L and sorted. Sorted cells were always >95% $CD4^{+}/mRFP^{+}/CD62L^{Lo}$.

In vitro suppression assay

In vitro suppression assays were performed as previously described (10).

GvHD grading

Mice were observed twice weekly for signs of GvHD using the previously described clinical scoring system (15).

Fluorescence microscopy

Animals were anesthetized with avertin and organs were imaged with a Zeiss SteREO Lumar V12 microscope with eGFP bandpass filter (Carl Zeiss MicroImaging, Inc., Thornwood, NY) as described (14).

Competitive T_{reg} migration assay

Competitive migration of WT versus CD62L −/− T_{reg} cells was done as described (14).

Histopathology

The sections were scored by one of us (A.P.-M.) who was blinded to the treatment given using a previously described method (14).

Quantitation of chemokine receptor transcripts

RNA was isolated from sort-purified T_{regs} using the Qiagen RNeasy Kit (Qiagen; Valencia, CA). Quantitative RT-PCR for chemokine receptor transcripts was performed using primers and probes to CCR1, CCR2, CCR4, CCR5, CCR7, CCR8, CCR9, CCR10, CXCR3 and CXCR4 (Applied Biosystems; Corvalis, OR). The ΔCt method was used to normalize transcripts to 18S RNA and to calculate fold induction.

Measurement of serum IFN-γ

Serum samples were obtained from mice receiving whole naïve T cells, with or without WT T_{regs}, $CD62L^{-/-}$ T_{regs}, $CD62L^{Lo}$ T_{regs} or BM only. The samples were recovered when animals reached a clinical GvHD score of 4. IFN-γ concentrations were determined according to the manufacturer's instructions using ELISA (Biolegend San Diego CA).

Statistical analysis

For GvHD scoring, we used Student's t-test; for overall survival we used Fisher's exact test, and for median survival we used the Mann–Whitney log rank test. p values ≤ 0.05 were considered significant.

Results

$CD62L^{-/-}$ T_{regs} mediate protection against lethal GvHD

To determine the precise requirement for CD62L expression in T_{reg}-mediated protection during GvHD, we isolated fresh $CD4^+/CD25^+$ cells from WT or CD62L deficient animals ($CD62L^{-/-}$). Unexpectedly, we did not observe a significant difference in the overall survival ($p = 1.0$) or median survival time ($p = 0.86$) in recipient mice given WT compared to $CD62L^{-/-}$ T_{regs} (Figure 1A). Both WT and $CD62L^{-/-}$ T_{regs} recipients had significantly improved overall survival ($p < 0.001$) compared to recipients of WT T cells alone. Our previous work has demonstrated that *in vitro* expanded $CD62L^{Lo}$ T_{regs} were unable to ameliorate GvHD pathology (5); however, our subsequent analysis of expanded $CD4^+/CD25^+$ cells has revealed considerable contamination by $FoxP3^-$ cells in the $CD62L^{Lo}$ fraction (M. Carlson, J. Serody; unpublished observation). We therefore isolated cells from

FIR mice in which the red fluorescent protein is expressed under control of the FoxP3 promoter (13), and thus, T_{regs} can be identified from the $CD4^+/CD25^+$ fraction by their expression of mRFP. Recipients of *ex vivo* expanded $mRFP^+/CD62L^{Lo}$ T_{regs} displayed only a very modest improved overall ($p = 0.09$) and median survival time ($p = 0.12$) relative to animals receiving T cells alone (Figure 1A). These results demonstrated that $CD62L^{-/-}$ T_{regs} were capable of providing protection from lethal acute and GvHD. These data also demonstrate that contamination of $CD62^{Lo}$ T_{reg} cells with effector cells was not an explanation for the lack of activity of $CD62L^{Lo}$ T_{regs} in the current study. The paucity of $CD62L^{Lo}$ T_{regs} present in FIR mice precluded the evaluation of this population of cells without *ex vivo* expansion.

Next, we determined disease severity using a defined clinical scoring system (15). Although the survival outcomes were not significantly different, WT T_{regs} did afford reduced clinical GvHD scores compared to $CD62L^{-/-}$ T_{regs} during the first 21 days posttransplant ($p < 0.04$ for days 7–21) (Figure 1B). Starting on day 24, and for the duration of the experiment, GvHD scores were not significantly different ($p > 0.05$) in recipients given either WT or $CD62L^{-/-}$ T_{regs}. Consistent with no improvement in overall or median survival, $CD62L^{Lo}$ T_{regs} did not reduce clinical manifestations of GvHD as compared to T cells alone (Figure 1B). Collectively, these data demonstrated that $CD62L^{-/-}$ T_{regs} were able to protect animals from lethal GvHD, albeit they did not suppress clinical GvHD manifestations as well as WT T_{reg} cells in the first 3 weeks posttransplant. In addition, $CD62L^{-/-}$ T_{regs} functioned more efficiently to prevent GvHD than $mRFP^+/CD62L^{Lo}$ T_{regs} posttransplantation.

$CD62L^{-/-}$ T_{regs} function normally to suppress T cell responses to allo-antigen in vitro

Because we observed significant differences early posttransplant in the clinical appearance of GvHD between recipients of WT and $CD62L^{-/-}$ T_{regs}, we sought to determine the ability of $CD62L^{-/-}$ T_{regs} to inhibit effector T cell responses to allo-antigen. To address this question, freshly isolated $CD4^+/CD25^+$ cells from WT and $CD62L^{-/-}$ mice were co-cultured with WT $CD4^+/CD25^-$ responder cells stimulated with irradiated B6D2 splenocytes. $CD62L^{-/-}$ and WT T_{regs} displayed equivalent suppression of WT effector T cells up to a 1:8 T_{reg}:Effector cell ratio (Figure 1C). Therefore, the early elevated GvHD scores of animals given $CD62L^{-/-}$ T_{regs} was not due to an intrinsic defect in their suppressive function. As described, $CD62L^{Lo}$ T_{regs} were potent suppressors of allo-reactive T cells *in vitro* up to a ratio of 1:32 T_{regs}: Effector cells (Figure S1).

GvHD target organ histopathology

Given the differences observed in clinical GvHD scores, we were interested in determining the impact that phenotypically different T_{regs} had on individual organ pathology. Histopathology scores in the colon were not statistically different between any of the groups (Figure 2A). Recipients of WT T_{regs} demonstrated less pathological damage in the lung as compared to recipients of $CD62L^{-/-}$ T_{regs} ($p = 0.05$) (Figure 2B). Examination of the liver demonstrated that both WT and $CD62L^{-/-}$ T_{regs} significantly inhibited GvHD pathology ($p < 0.03$) compared to recipients of effector T cells alone (Figure 2C). Interestingly, despite the modest difference in tissue pathology, there were significant differences in serum IFN-γ levels in mice given effector T cells alone compared to WT or $CD62L^{-/-}$ T_{regs} ($p < 0.01$) (Figure 2D). Overall, these results demonstrated that with the exception of worsened lung pathology, $CD62L^{-/-}$ T_{regs} functioned as well as WT T_{regs} to prevent GvHD, while both were potent in their ability to inhibit systemic IFN-γ production.

$CD62L^{-/-}$ T_{regs} traffic to secondary lymphoid tissues and GvHD target organs

Because we observed differences early on in the clinical manifestation of GvHD between recipients of WT and $CD62L^{-/-}$ T_{regs}, we were interested in determining the trafficking

pattern of these T_{regs}. To evaluate *in vivo* T_{reg} trafficking, we used a competitive lymphocyte migration assay (16). As illustrated in Figure 3A, 6 days after transfer, $CD62L^{-/-}$ T_{regs} were found at a similar frequency as WT T_{regs} in the liver, lung, spleen, bone marrow and mesenteric lymph node (MLN), although as expected, there were substantially fewer $CD62L^{-/-}$ T_{regs} in the PLNs of recipient animals. Further analysis 16 days post-T_{reg} transfer showed no difference between WT and $CD62L^{-/-}$ T_{reg} migration to liver, spleen, bone marrow or MLN (Figure 3B). However, although not statistically different, there were fewer $CD62L^{-/-}$ T_{regs} detected in the lung and PLN on day 16 compared to WT T_{regs}, which correlated with the enhanced GvHD in the lung of recipient animals receiving $CD62L^{-/-}$ T_{regs} (Figure 3B).

To confirm our findings regarding the function of CD62L in the migration of T_{regs} in a lymphopenic environment, we performed *in vivo* imaging using fluorescence stereomicroscopy. In the MLN(Figure 3C) and spleen (Figure 3D)we found similar distribution and GFP signal intensity by WT and $CD62L^{-/-}$ T_{regs}, indicating that the migration and accumulation of $CD62L^{-/-}$ T_{regs} was indistinguishable from WT T_{regs} in these organs 16 days posttransplantation. Interestingly, we observed fewer GFP^{+} $CD62L^{-/-}$ T_{regs} in the lung (Figure 3E) and PLN (Figure 3F) at this time point. Taken together, these observations illustrated that $CD62L^{-/-}$ T_{regs} home to GvHD target organs, similar to WT T_{regs}, with the exception of a modest impairment in migration to the lung. Differences in the migration of WT compared to $CD62L^{-/-}$ T_{regs} to PLNs were found in the first week posttransplantation demonstrating the importance of CD62L in the initial migration of T_{regs} to PLNs. However, at day 16 these differences were minimized indicating that CD62L was not absolutely required for the eventual migration of T_{regs} to PLNs.

$CD62L^{Hi}$, $CD62L^{-/-}$ and $CD62L^{Lo}$ T_{regs} display differential expression of chemokine receptors

The finding of $CD62L^{-/-}$ T_{regs} in the PLNs of irradiated recipients was somewhat surprising given the role that CD62L plays in T cell rolling and homing to lymph nodes. This observation suggests that in the absence of CD62L other proteins important for T cell migration may serve a similar function. To this end we examined the phenotypic profile of T_{regs} based on CD62L expression (Figures 4A–C). As shown in Figure 4D, $CD62L^{Hi}$ and $CD62L^{-/-}$ T_{regs} have increased expression of CCR7 mRNA relative to $CD62L^{Lo}$ T_{regs}. We then compared the three T_{reg} types to naïve $CD4^{+}$/$mRFP^{-}$ cells in their mRNA expression of other chemokine receptors. $CD62L^{-/-}$ T_{regs} resembled $CD62L^{Lo}$ T_{regs} in the expression of CCR2, CCR4 and CXCR3, and resembled $CD62L^{Hi}$ T_{regs} in the expression of CCR9. $CD62L^{-/-}$ T_{regs} had intermediate levels of CCR5 and CCR8, with distinctive expression of CCR1 and CCR10. Collectively, these data demonstrated that the migratory profile of $CD62L^{-/-}$ T_{regs} was that of an intermediate activated phenotype with higher expression of pro-inflammatory chemokine receptors compared to $CD62L^{Hi}$ T_{regs} and much greater expression of CCR7 compared to $CD62L^{Lo}$ T_{regs}.

Discussion

In the current work, we were interested in determining whether CD62L itself was critical for T_{reg} function and migration into lymphoid tissue. We demonstrated that CD62L was not critical for T_{reg} function to prevent GvHD lethality as $CD62L^{-/-}$ T_{regs} afforded substantial protection from lethal acute GvHD in the clinically relevant model employed. WT T_{regs} yielded reduced clinical scores compared to $CD62L^{-/-}$ T_{regs} during the first 3 weeks posttransplant, which correlated with delayed migration of $CD62L^{-/-}$ T_{regs} to PLNs. Histopathological analysis of GvHD target organs correlated with the clinical scores, as recipients of WT T_{regs} showed improved pathology in the lung and similar pathology in the colon and liver compared to $CD62L^{-/-}$ T_{regs}. Lastly, we demonstrated differential

chemokine receptor expression of T_{regs} based on CD62L expression, where the $CD62L^{-/-}$ T_{regs} displayed a phenotype that appeared to be an intermediate between the naïve $CD62L^{Hi}$ and activated $CD62L^{Lo}$.

Previous reports examining the role of CD62L in T_{reg}-mediated inhibition of GvHD suggested either that (1) CD62L itself was critically important in the function of T_{regs} or that (2) the $CD62L^{Hi}$ phenotype functioned differently than $CD62L^{Lo}$ T_{regs} but that CD62L itself was not critical (5,9). Our data demonstrated that CD62L itself was not critically required for the prevention of GvHD lethality or for the ability to migrate into LN post transplantation. While there was no difference in either overall or median survival time, our data indicated that CD62L did serve as an accessory molecule given the statistical difference in clinical scores between WT and $CD62L^{-/-}$ T_{regs} during the first 3 weeks posttransplantation. It is of interest that we also observed no statistical difference in clinical scores between $CD62L^{-/-}$ and $CD62L^{Lo}$ T_{regs} for the first 2 weeks posttransplantation suggesting CD62L serves early on to promote T_{reg} inhibition of GvHD most likely by enhancing the migration of T_{regs} into lymphoid tissue.

One concern in our previous studies in which we expanded $CD4^+/CD25^+$ T cells to obtain a $CD62L^{Lo}$ population was the difficulty in eliminating $CD25^+$ effector cells from the T_{reg} infusion (5). Here, we have circumvented this concern by using T_{regs} from FIR mice in which mRFP is under control of the FoxP3 promoter and thus cells expressing FoxP3 can be detected using flow cytometry (13). Our data confirm previous observations that $CD62L^{Lo}$ T_{regs} were not sufficient to prevent GvHD in the overwhelming majority of transplanted recipients. The possibility of impaired suppressive function of these cells was ruled out by *in vitro* analysis in which $CD62L^{Lo}$ T_{regs} were more proficient suppressors of T cell responses to allo-antigen, consistent with the previously published data (17). Therefore, the inability of $CD62L^{Lo}$ T_{regs} to provide protection against GvHD could not be explained by impaired function but may be due to impaired homing to lymphoid tissue or diminished survival after infusion.

Examination of the pathology in individual organs revealed that WT and $CD62L^{-/-}$ T_{regs} ameliorated disease in the liver, whereas WT T_{reg} recipients displayed reduced pathology in the lung as compared to $CD62L^{-/-}$ T_{reg} recipients. The increased lung pathology correlated with modestly impaired $CD62L^{-/-}$ T_{reg} migration to the lung. The accumulation of IFN-γ in the serum has been shown to be a predictor of GvHD mortality (18). We also documented a substantial reduction in the level of IFN-γ in the serum of animals receiving either WT or $CD62L^{-/-}$ T_{regs} an effect not seen in recipients of $mRFP^+/CD62L^{Lo}$ T_{regs} (data not shown). Here again, a functional distinction was made between $CD62L^{-/-}$ and $CD62L^{Lo}$ T_{regs}.

While it is clear that T_{regs} do inhibit effector T cell expansion and suppress effector functions, it is less clear as to whether the inhibition is in lymphoid tissues or GvHD target organs. In the current report, we demonstrated that $CD62L^{-/-}$ T_{regs} migrate to GvHD target organs with similar efficiency as WT T_{regs}; however, their accumulation within the PLNs was delayed. It is interesting to note that this delay corresponded with increased clinical GvHD scores, thus supporting the hypothesis that entry into lymph nodes by T_{regs} was important in inhibiting the initial expansion of donor T cells. The inability of $CD62L^{Lo}$ T_{regs} to inhibit GvHD has been attributed to ineffective trafficking to secondary lymphoid tissues (9). Normal trafficking seen in $CD62L^{-/-}$ T_{regs} provides another distinction between $CD62L^{Lo}$ and $CD62L^{-/-}$ phenotypes.

Other studies have examined chemokine receptor expression on T_{reg} subsets, including the $CD62L^{Hi}$ and $CD62L^{Lo}$ populations (19,20). Our data are in agreement that the $CD62L^{Hi}$ fraction expressed high levels of the lymph node homing chemokine receptor CCR7. Of

interest, the $CD62L^{-/-}$ population also expressed high levels of CCR7, providing a plausible mechanism for their migration to secondary lymphoid tissues. In keeping with an activated status the $CD62L^{Lo}$ T_{regs} expressed high levels of CCR5 and CCR8 while the $CD62L^{-/-}$ T_{regs} displayed intermediate expression.

In summary, our data demonstrate that posttransplant, CD62L was dispensable for T_{reg} inhibition of GvHD lethality.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

Allo-HSCT	allogeneic hematopoietic stem cell transplantation
APC	antigen presenting cell
eGFP	enhanced green fluorescent protein
GvHD	graft-versus-host disease
GvL	graft-versus-leukemia
PLN	peripheral lymph nodes
MLN	mesenteric lymph node
TCD BM	T cell depleted bone marrow
T_{regs}	regulatory T cells
WT	wild type.

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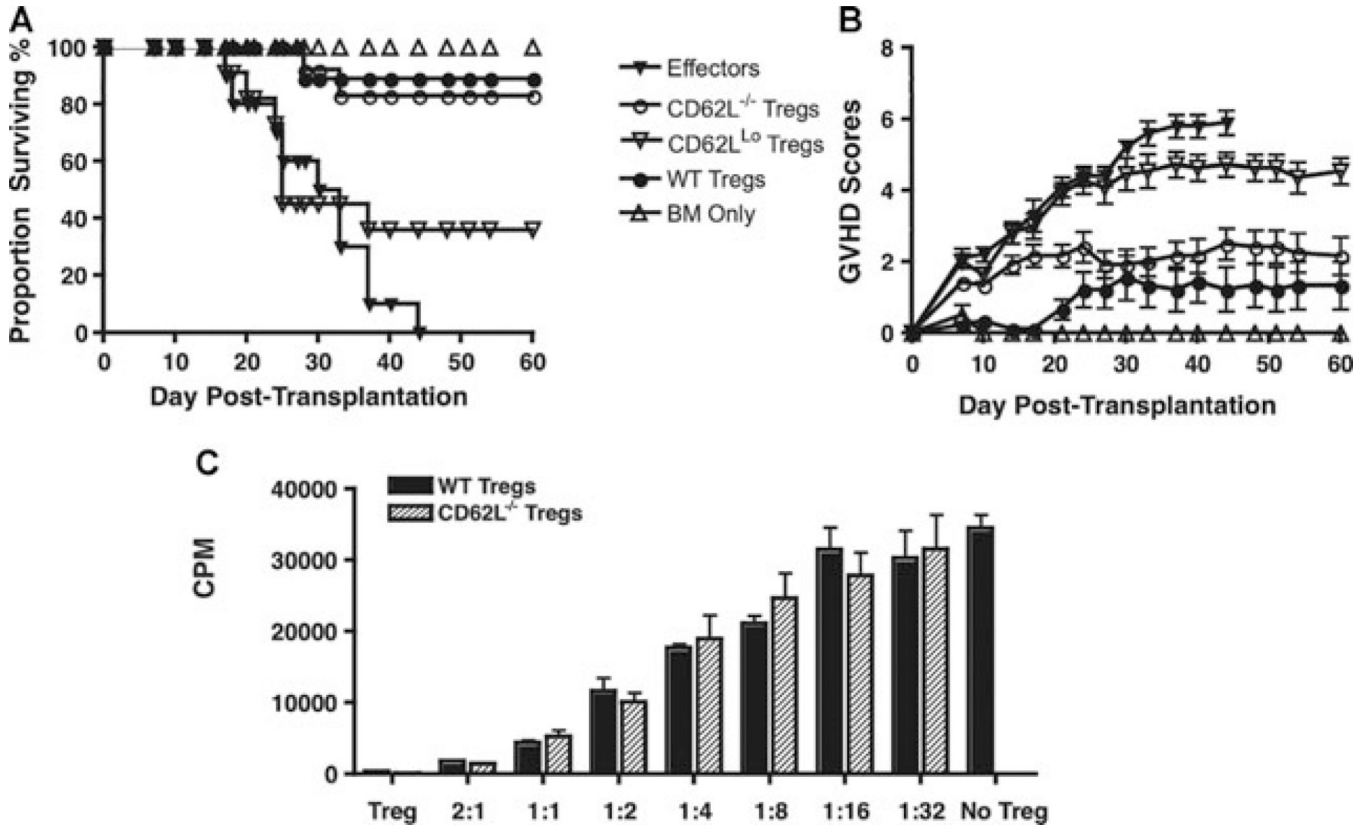

Figure 1. CD62L$^{-/-}$ T$_{regs}$ protect from lethal acute GvHD and are potent suppressers *in vitro*

1.25 × 10^6 WT (of which approximately 80% expressed CD62L and 67% had high levels of expression of CD62L), CD62L^{Lo} T$_{regs,}$ or CD62L$^{-/-}$ T$_{regs}$ were transferred with 3 × 10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 4 × 10^6 whole splenic T cells from WT mice were then transferred on day +2 (n = 9 WT T$_{regs}$, n = 11 CD62L^{Lo} T$_{regs,}$ n = 12 CD62L$^{-/-}$ T$_{regs}$, n = 10 Effectors alone, n = 4 BM only). Animals were monitored for (A) survival and (B) signs of GvHD. Data represent mean score ± SEM at each time point. (C) Suppression of WT responder cell (CD4$^+$/CD25$^-$) proliferation in response to B6D2 alloantigen by WT (■) or CD62L$^{-/-}$ T$_{regs}$ (▨) was determined as described in the Methods section.

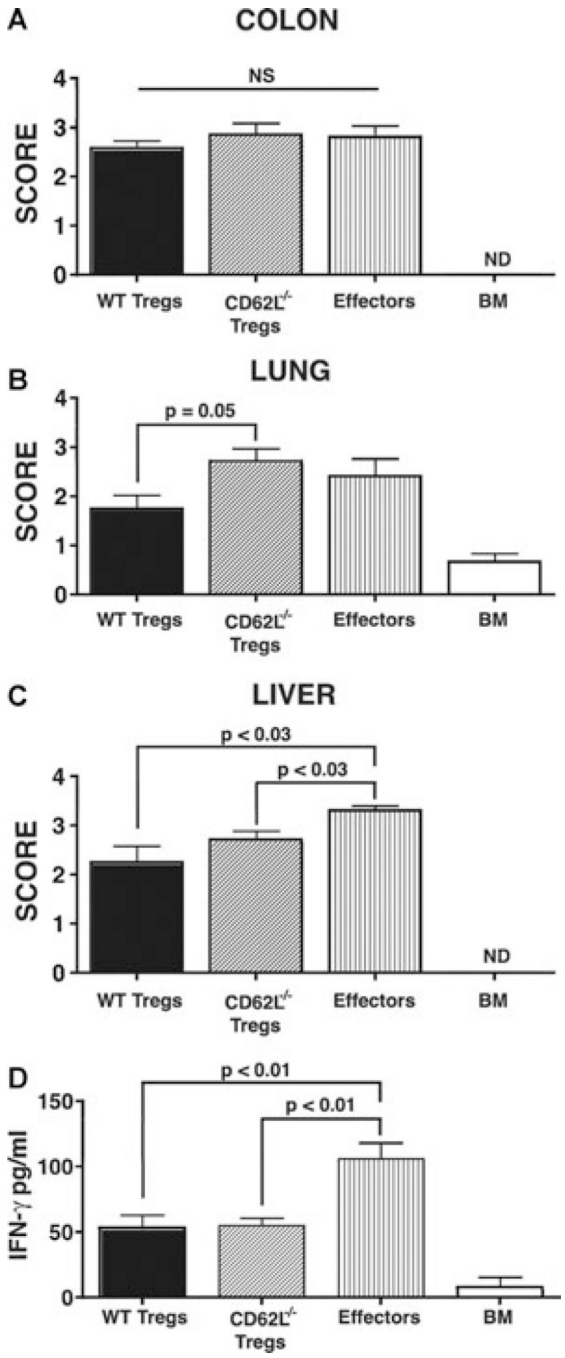

Figure 2. CD62L$^{-/-}$ and WT T_{regs} suppress liver pathology

1.25×10^6 WT or CD62L$^{-/-}$ T_{regs} were transferred with 3×10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 4×10^6 whole splenic T cells from WT mice were then transferred on day +2 (n = 6 WT T_{regs} (■), n = 7 CD62L$^{-/-}$ T_{regs}, (▩), n = 8 Effectors alone (▩), n = 3 BM only (□). Animals were recovered when clinical scores reached a total of >4. Animals that did not reach a score of 4 were recovered on days 25–27 posttransplant. Histopathological assessment of the (A) colon, (B) lung, (C) liver. (D) Serum was recovered from animals at the time of histopathology assessment and analyzed by ELISA for levels of IFN-γ.

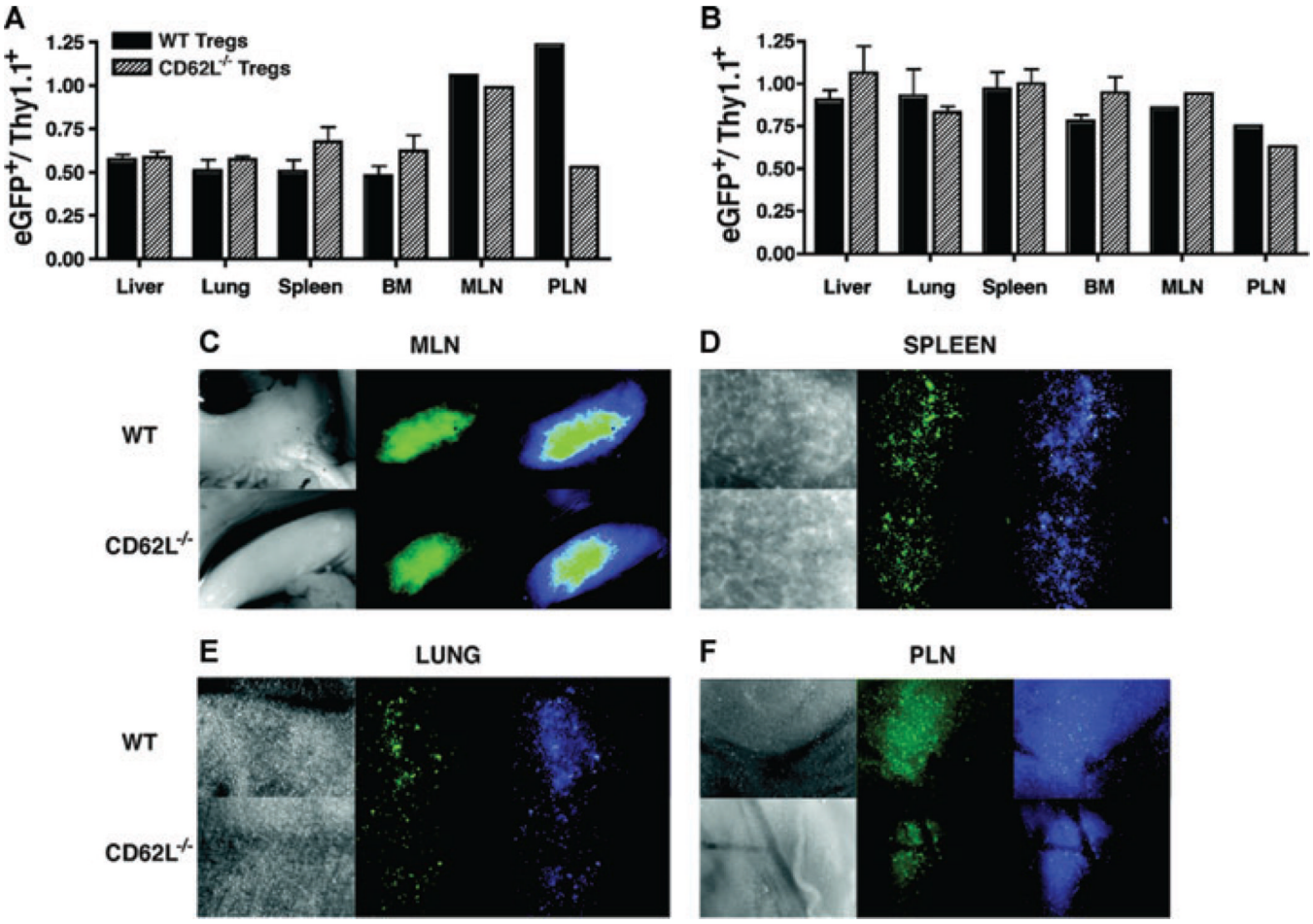

Figure 3. CD62L$^{-/-}$ and WT T_{reg} trafficking

5×10^5 WT-GFP (■) or CD62L$^{-/-}$-GFP (▨) T_{regs} were transferred with 5×10^5 Thy1.1$^+$ T_{regs} along with 3×10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 2×10^6 whole splenic T cells from WT mice were then transferred on day +2. On days 6 (A) and 16 (B) post-T_{reg} transfer, lymphocytes were isolated from GvHD target organs and secondary lymphoid tissues as described in the Methods section. The ratio of eGFP$^+$/FoxP3$^+$:Thy1.1$^+$/FoxP3$^+$ cells are shown (n = 4 for each group). N = 4 animals/time point (MLNs were pooled for each group, and PLNs were pooled for each group). 1.0×10^6 WT-GFP (*top*) or CD62L$^{-/-}$-GFP (*bottom*) T_{regs} were transferred along with 3×10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 2×10^6 whole splenic T cells from WT mice were then transferred on day +2. 16 days post-T_{reg} transfer animals were anesthetized with avertin and organs were imaged with a Zeiss SteREO Lumar.V12 microscope with eGFP bandpass filter. Brightfield images (*left*), and GFP images (*middle*) were taken for each organ. GFP intensities (*right*) were determined by software analysis. (C) MLN (D) spleen (E) lung (F) peripheral lymph node. Original magnification: lung = 25X, spleen = 40X, MLN = 45X, ILN = 45X. Data represent mean score ± SEM for each organ.

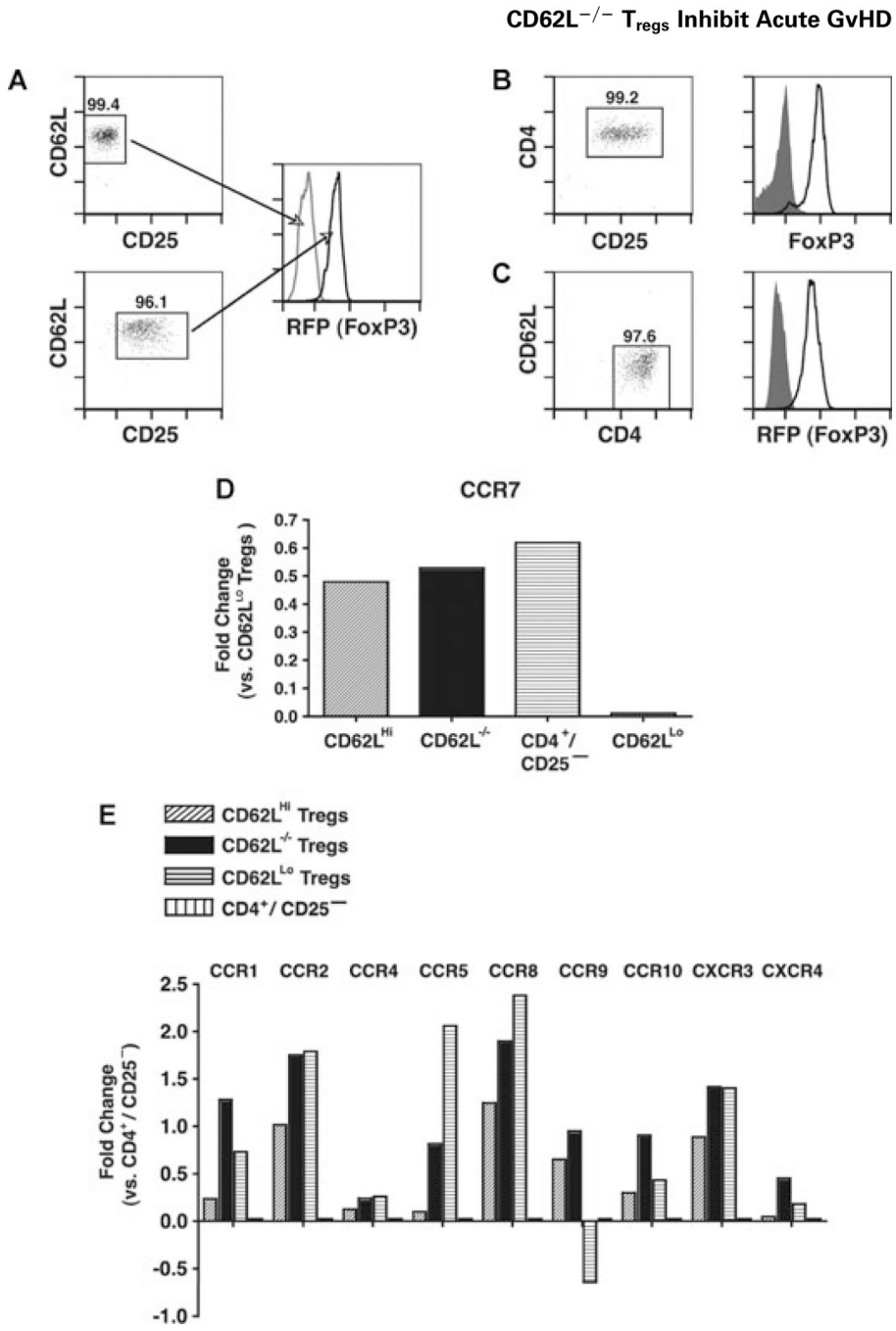

Figure 4. T_{reg} chemokine receptor expression based on CD62L expression

Sort purification of cells for quantitative real-time PCR analysis. (A) $CD4^+/CD25^+/mRFP^+/CD62L^{Hi}$ T_{regs} (*bottom*) and $CD4^+/CD25^-/mRFP^-/CD62L^{Hi}$ naïve T cells (*top*) were sort purified from mRFP-FoxP3 mice. (B) $CD4^+/CD25^+$ T_{regs} were sort purified from $CD62L^{-/-}$ mice. (C) $CD4^+/mRFP^+/CD62L^{Lo}$ T_{regs} were sort purified following *in vitro* expansion. RNA was extracted and real-time PCR performed as described in the Methods section. (D) CCR7 expression on $mRFP^+/CD62L^{Hi}$ T_{regs} (▨), $CD4^+/CD25^+/CD62L^{-/-}$ T_{regs} (■), naïve $CD4^+/CD25^-$ T cells (▤) and $mRFP^+/CD62L^{Lo}$ Tregs (▩). Data are shown as relative change in expression (logarithmic scale) compared to $CD4^+/mRFP^+/CD62L^{Lo}$ T_{regs}. (E) Chemokine receptor expression on $mRFP^+/CD62L^{Hi}$ T_{regs} (▨), $CD4^+/CD25^+/CD62L^{-/-}$

T_{regs} (■), $mRFP^+/CD62L^{Lo}$ T_{regs} (▤) and naïve $CD4^+/CD25^-$ T cells (▥). Data are shown as relative change in expression (logarithmic scale) compared to naïve $CD4^+/CD25^-$ T cells. Data are representative of three independent experiments.